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European Journal of Pharmacology 505 (2004) 93–101

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Passage of erythropoietic agents across the blood–brain barrier: a comparison of human and murine erythropoietin and the analog darbepoetin alfa

William A. Banks[a,*], Nelson L. Jumbe[b], Catherine L. Farrell[b], Michael L. Niehoff[a], Anne C. Heatherington[b]

[a]*Geriatrics Research, Education, and Clinical Center, Veterans Affairs Medical Center-St. Louis and Saint Louis University School of Medicine, Division of Geriatrics, Department of Internal Medicine, United States*

[b]*Amgen Inc., Thousand Oaks, CA, United States*

Received 5 October 2004; accepted 12 October 2004

Abstract

Studies have suggested that erythropoietin (EPO) may be used to treat stroke in both animals and humans. It is thought to exert its effects directly on the brain and studies with therapeutic doses have shown that it can cross the blood–brain barrier. Here, we compared in a blinded fashion the ability of three erythropoietic agents (murine erythropoietin, human erythropoietin, and darbepoetin alfa, an analog of human erythropoietin in clinical use) to cross the blood–brain barrier of the mouse. High-performance liquid chromatography (HPLC) results showed that all three erythropoietic agents were enzymatically resistant in brain and blood. The unidirectional blood-to-brain influx rates (K_i) as measured by multiple-time regression analysis showed that all the erythropoietic agents crossed the blood–brain barrier at about the same rate as albumin, suggesting that they cross the blood–brain barrier by way of the extracellular pathways. No saturable component to influx was found, but indirect evidence suggested a brain-to-blood efflux system. The percent of the intravenously injected dose taken up per gram of brain (%Inj/g) ranged from 0.05 to 0.1 %Inj/g among the three erythropoietic agents and peaked about 3 h after IV injection. For other substances, this range of %Inj/g is known to produce direct effects on brain function. We conclude that erythropoietic agents cross the blood–brain barrier by way of the extracellular pathways in amounts that are likely sufficient to explain their neuroprotective effects.

Keywords: Brain; Pharmacokinetics; Drug delivery; Blood–brain barrier; Erythropoietin; Stroke; Neuroprotection

1. Introduction

Erythropoietin (EPO) is a cytokine glycoprotein produced by the kidney in response to hypoxia. Erythropoietin may have neuroprotective effects that are independent of its erythropoietic actions. Erythropoietin is neuroprotective in vitro and is effective in treating stroke when given either directly into brain or peripherally in animals (Marti et al., 2000; Alafaci et al., 2000). In humans, erythropoietin significantly improves outcome when peripherally administered within 6 h of stroke, a time period too short to induce an increase in hemoglobin, and under a regimen which has no effect on erythrocyte levels (Ehrenreich et al., 2002). Erythropoietin appears to be neuroprotective in vivo against injuries induced in animals by blunt trauma, hypoglycemia, experimental allergic encephalomyelitis, kainate-induced seizures, neuronal depolarization, subarachnoid hemorrhage, and conditions resulting in low ATP levels (Alafaci et al., 2000). Finally, desialylation produces an erythropoietin that although unable to increase hemoglobin still appears to be neuroprotective (Erbyraktar et al., 2003).

Investigators believe that erythropoietin acts directly on brain to produce its effects. Receptors for erythropoietin are

* Corresponding author. 915 North Grand Boulevard, St. Louis, MO 63106, United States. Tel.: +1 314 289 7084; fax: +1 314 289 6374.
E-mail address: bankswa@slu.edu (W.A. Banks).

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doi:10.1016/j.ejphar.2004.10.035

found throughout the brain (Brines et al., 2000; Marti et al., 1996). These receptors are upregulated in the ischemic penumbra after stroke (Marti et al., 2000). Erythropoietin has neuroprotective effects which may be mediated in part by limiting damage done by the hypoxic-induced release of glutamate and nitric oxide (Marti et al., 2000).

For peripherally administered erythropoietin to exert its neuroprotective effects directly on neurons, it would have to cross the blood–brain barrier. In general, the blood–brain barrier prevents large proteins from entering the CNS (Davson and Segal, 1996). However, the blood–brain barrier has been shown to act more as a regulatory interface than an absolute barrier to cytokines by excluding some and transporting others (Banks et al., 1995). Several studies have failed to find an elevation in levels of erythropoietin in the CSF after peripheral administration of erythropoietin (Juul et al., 1997; Juul et al., 1999; Marti et al., 1997). However, increases in CSF erythropoietin levels have been found after giving therapeutically effective doses to humans (Ehrenreich et al., 2002) and animals (Brines et al., 2000; Alafaci et al., 2000). Additionally, the brain capillaries which comprise the blood–brain barrier possess receptors for erythropoietin (Brines et al., 2000), suggesting that the blood–brain barrier might have a saturable transport system for erythropoietin as it does for other cytokines, such as leptin (Banks et al., 1996), interleukin-1 (Banks et al., 1991), and tumor necrosis factor alfa (Gutierrez et al., 1993).

Here, we characterized in mice the permeabilities of the blood–brain barrier to three radioactively labeled erythropoietic agents which bind to the erythropoietin receptor (Egrie et al., 2003; Elliot et al., 2003). We hypothesized that species differences in blood–brain barrier permeation could occur so we studied both human and mouse erythropoietin. We also studied a longer acting erythropoietic agent in clinical use, darbepoetin alfa to compare its blood–brain barrier permeation to that of human erythropoietin and murine erythropoietin.

2. Methods

2.1. Radioactive labeling and purification of erythropoietic agents and albumin

Erythropoietic agents provided by Amgen (Thousand Oaks, CA) were coded “A”, “B”, or “C” so that the investigators were not aware which of the erythropoietic agents were being studied. Erythropoietic agents were radioactively labeled with ^{131}I in iodogen pre-coated tubes. Tris–HCl (100 μl of 25 mM in 0.4 M NaCl at pH 7.5) was added to an iodogen tube and gently mixed with 2 mCi of ^{131}I (2 μl). After 6 min of incubation with occasional gentle mixing, this solution was transferred from the iodogen tube to a new glass tube and 5 μg of an erythropoietin added. This solution was incubated for 9 min with occasional gentle mixing. L-Tyrosine (50 μl of a 10 mg/ml solution in Tris–HCl pH 7.5 buffer) was added to react with unincorporated ^{131}I. After 5 min of incubation with occasional gentle mixing, 100 μl of the phosphate buffer (pH 7.5) was added and the entire solution placed on a column of G-10 sephadex. The radioactively labeled erythropoietic agent (I-human erythropoietin, I-murine erythropoietin, I-darbepoetin alfa) was eluted in 0.1 ml fractions with the phosphate buffer solution. All purified I-erythropoietic agents were more than 95% precipitable with acid and eluted as monopeaks by high-performance liquid chromatography (HPLC; see Section 3.1). No study was done with an I-erythropoietic agent that had acid precipitation which had decreased more than 10% from the original value. After correction for counting efficiency of the gamma counter, specific activity was estimated to range between 150 and 200 Ci/g. Human serum albumin was labeled with ^{125}I (I-Albumin) by the chloramine-T method and purified on a column of G-10.

I-Darbepoetin alfa was compared to unlabeled darbepoetin alfa for biological activity in a cell line transfected with the erythropoietin receptor in a 10-point standard curve over the concentration range of 62.5 pg/ml to 16 ng/ml. All curves were run in triplicate and two lots of I-darbepoetin alfa were tested. Proliferation of the murine 32D cell line transfected with human erythropoietin receptors, designated as 32Dcl3.sccl2 (Migliaccio et al., 1989), in response to I-darbepoetin alfa and darbepoetin alfa was measured by incorporation of [methyl-^{3}H]-thymidine.

2.2. Measurement of blood-to-brain uptake

All animal experiments were done in accordance with internationally accepted standards under protocols approved by the local animal care committee and in an approved institution. Multiple-time regression analysis (Blasberg et al., 1983; Patlak et al., 1983) was used to measure the unidirectional influx rate (K_i). Male CD-1 mice from our colony were anesthetized with ethyl carbamate and the right carotid artery and left jugular vein exposed. Mice were given an injection into the jugular vein of 0.2 ml of lactated Ringer’s solution containing 1% bovine serum albumin, 5×10^5 cpm of a radioactively labeled erythropoietic agent, and 5×10^5 cpm of I-Albumin. Blood from the carotid artery was obtained at 2, 5, 10, 30, 60, 120, 240, 480, or 1440 min after i.v. injection, centrifuged for 10 min at 5000×g at 4 °C, and the radioactivity level in the serum determined. This experiment was repeated in another group of mice with or without inclusion of I-Albumin. In this repeat experiment, time points up to 48 h were included, but analysis was limited to 24 h because degradation products accounted for more than 50% of radioactivity at the later time points. Immediately after collection of the arterial blood, the mouse was decapitated and the level of the radioactivity in the whole brain (with the pituitary and the pineal gland removed) determined. The brain/serum ratios for the radioactive erythropoietic agent and I-Albumin in units of

microliters per gram [(cpm/g of brain)/(cpm/μl serum)] were plotted against exposure time (Expt):

$$\text{Expt} = \left[\int_0^t \text{Cp}(\tau)\text{d}\tau\right]/\text{Cpt} \quad (1)$$

where Cpt is the level of radioactivity in serum at time t (Blasberg et al., 1983; Patlak et al., 1983). The slope of the linear portion of the relation between the brain/serum ratios and Expt was calculated by the least squares method and measures the unidirectional influx rate (K_i) from blood to brain and is reported with its standard deviation of the residuals.

2.3. Percent of injected dose taken up by brain

First, the cpm/ml of serum was divided by cpm injected and multiplied by 100 to yield the percent of the i.v. injected dose present in each ml of serum (%Inj/ml). The half-life clearance of radioactivity from blood was calculated by multiplying by 0.301 the inverse of the slope for the relation of log(%Inj/ml) vs. time. The percent of the i.v. injected dose taken up per gram of brain (%Inj/g) for time t was calculated by the equation:

$$\%\text{Inj/g} = (R - 10)(\%\text{Inj/ml})/1000 \quad (2)$$

where R is the brain/serum ratio in microliter per gram at time t, %Inj/ml is the percent of the i.v. injected dose present in 1 ml of serum at time t, and the value 10 is used to correct for the vascular space of brain. Times of less than 30 min were not used as retrospective analysis showed that scatter was large and included many negative numbers (i.e., brain/serum ratios below 10). Additionally, the mean values for the 60 min value for I-darbepoetin alfa and the 120 min value for I-human erythropoietin were negative and so excluded from analysis. The area under the curve for %Inj/g vs. time for the first 8 h after i.v. injection was computed with the Prism 3.0 program (GraphPad, San Diego, CA) which uses the trapezoidal method.

2.4. Capillary depletion

The capillary depletion method was used to determine the extent to which the radioactive erythropoietic agents were retained by capillaries as opposed to crossing the full width of the capillary wall to enter the brain parenchymal (cellular and interstitial fluid) space. The method of Triguero et al. (1990) as modified for mice by Gutierrez et al. (1993) was used. After anesthesia, mice received an injection into the jugular vein of 0.2 ml of lactated Ringer's with 1% bovine serum albumin containing 1×10^6 cpm of the radioactive erythropoietic agent and 1×10^6 cpm of I-Albumin. At 10 min or 24 h later, the abdomen was opened and blood was collected from the abdominal aorta. The thorax was opened, the thoracic descending aorta clamped, the left and right jugular veins severed, and the brain flushed of its intravascular contents by injecting 20 ml lactated Ringer's solution over 1 min into the left ventricle of the heart. The mouse was decapitated and the brain harvested. The cerebral cortex was isolated and weighed and placed in ice-cold physiologic buffer (10 mM HEPES, 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, and 10 mM D-glucose adjusted to pH 7.4). The cortex was then homogenized using a glass tissue grinder (10 strokes) in 0.8 ml physiologic buffer. Dextran solution, 1.6 ml of a 26% solution in physiologic buffer, was added to the homogenate, mixed vigorously, and homogenized (three strokes). The homogenate was centrifuged at $5400\times g$ for 15 min at 4 °C in a swing bucket rotor. The pellet which contains the brain vasculature and the supernatant which contains the brain parenchyma were carefully separated and the radioactivity of each component determined using a gamma counter. The parenchyma/serum and capillary/serum ratios (μl/g) were calculated by the equation:

$$\text{Ratio} = (\text{cpm Fr})/(w)(\text{cpm/μl serum})$$

where cpm Fr is the cpm in either the parenchyma or vasculature fraction, w is the weight of the cortex, and cpm/μl serum is the level cpm in a microliter of serum.

2.5. Identification of radioactivity in brain and serum

Carotid artery blood and brain samples were collected 15 min, 2 h, and 24 h after the i.v. injection of an I-erythropoietic agent (biological samples). Serum was obtained by centrifugation and lyophilized. Brains were homogenized in ice-cold Ringer's lactated solution containing 1% bovine serum albumin and the supernatant lyophilized. Samples were reconstituted in distilled water and injected onto HPLC. The elution gradient began as 70% of solution A (0.1% trifluoroacetic acid in distilled water) and 30% of solution B (0.1% trifluoroacetic acid in acetylnitrile). Between 0.1 and 20 min, the gradient was linearly increased to 100% of solution B. After 5 min, the gradient was linearly decreased over a 10 min period back to 70% of solution A.

To determine the amount of degradation occurring during processing, radioactivity was placed in the bottom of test tubes and brains and whole blood collected from mice who had not received i.v. injections of radioactive erythropoietic agents (processing controls). These samples were processed as above and their results taken as 100%. The percent of radioactivity eluting as an intact erythropoietic agent in a biological sample was divided by the percent of radioactivity eluting in the respective processing control. The log of the resulting number was plotted against time and the half-time degradation rate calculated based on regression analysis.

2.6. Statistical analysis

Regression lines were calculated by the least squares method and compared statistically with Prism 3.0 (GraphPad,

Table 1
Time taken (in hours) for 50% of the radioactivity recoverable from serum or brain to represent degradation products (see Identification of radioactivity in brain and serum)

	Serum (h)	Brain (h)
I-Murine erythropoietin	20	8.66
I-Human erythropoietin	26.2	47.6
I-Darbepoetin alfa	ND	34.8

ND=no degradation measurable. Results are based on HPLC results from two animals/time point at three time points (n=6); see Figs. 1 and 2 for representative HPLCs for most stable (I-darbepoetin alfa in serum and I-murine erythropoietin in brain).

San Diego, CA). Regression lines are reported with their slopes±their standard errors of the mean, correlation coefficients (r), and the number of mice used per line (n). Means are reported with their standard errors and n. Two means were compared by Student's t-test and more than two groups were compared by analysis of variance (ANOVA) followed by Newman–Keuls post-test.

3. Results

3.1. HPLC

With time, an increasing amount of radioactivity represented degradation products rather than intact I-erythropoietic agent. The least stable was I-murine erythropoietin in brain and the most stable was I-darbepoetin alfa in serum (Table 1; Figs. 1 and 2). Table 1 shows the results for calculations of half-time degradation; that is, the time taken for 50% of the radioactivity recovered from brain or serum to represent radioactive degradation products. Subsequent analysis was limited to those times when more than 50% of the radioactivity represented intact material.

3.2. Murine erythropoietin

I-murine erythropoietin was the least stable of the three erythropoietic agents in both serum and brain (Table 1; Figs. 1 and 2). Analysis was limited to 8 h, since the radioactivity in brain at subsequent time points eluted by HPLC as less than 50% I-murine erythropoietin (Fig. 1). The K_i for I-murine erythropoietin (Fig. 3; Table 2) was 11.1±2.08 nl/g min (n=13, r=0.853, P<0.001, where P refers to a statistically significant correlation between brain/serum ratios and exposure time). It was noted that brain/serum ratios for time points beyond 8 h for I-murine erythropoietin departed from linearity, which would have excluded them from mutliple-time regression analysis. A repeat study, injected without I-Albumin and analyzed to 8 h, gave a K_i of 12.9±3.16 nl/g (n=11, r=0.806, P<0.005). The K_i for the co-injected I-Albumin was 1.83±0.31 nl/g min (n=17, r=0.836, P<0.001) when analyzed to 24 h. When confined to the first 8 h, no significant relation existed between the brain/serum ratios and Expt for I-Albumin, indicating that no

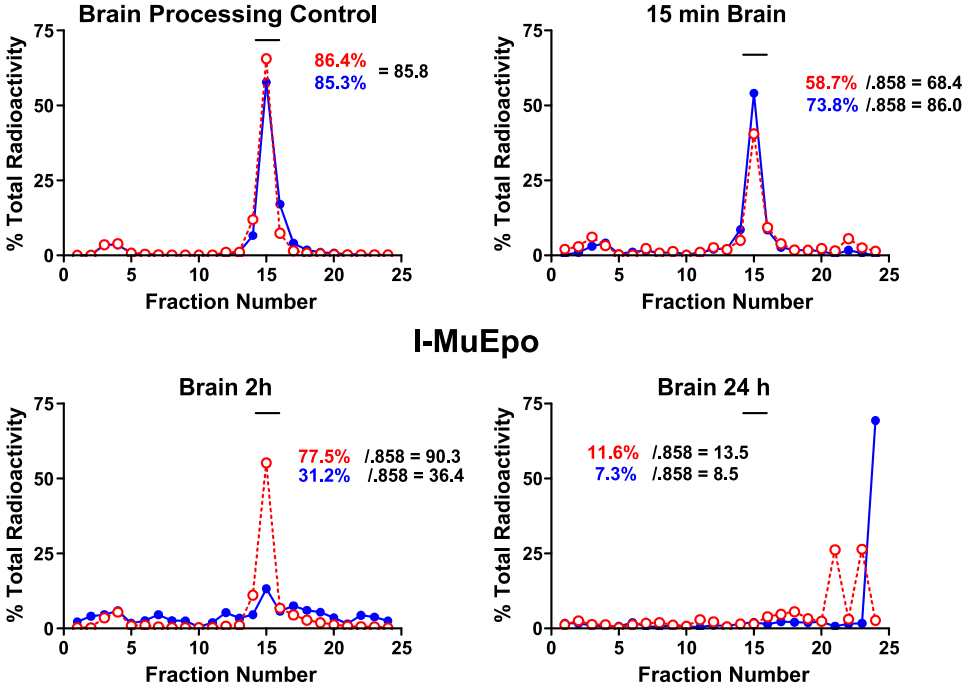

Fig. 1. HPLC results for I-murine erythropoietin: This compound in the brain showed the most rapid accumulation of degradation products. Radioactivity recovered from brain 15 min, 2 h, and 24 h after i.v. injection of I-murine erythropoietin (I-MuEPO). The numbers to the right of the peaks shows the percent of material eluting in the region of I-murine erythropoietin with the upper numbers correlating with the open symbol/broken line chromatograph and the lower numbers correlating to the closed symbol/solid line chromatograph.

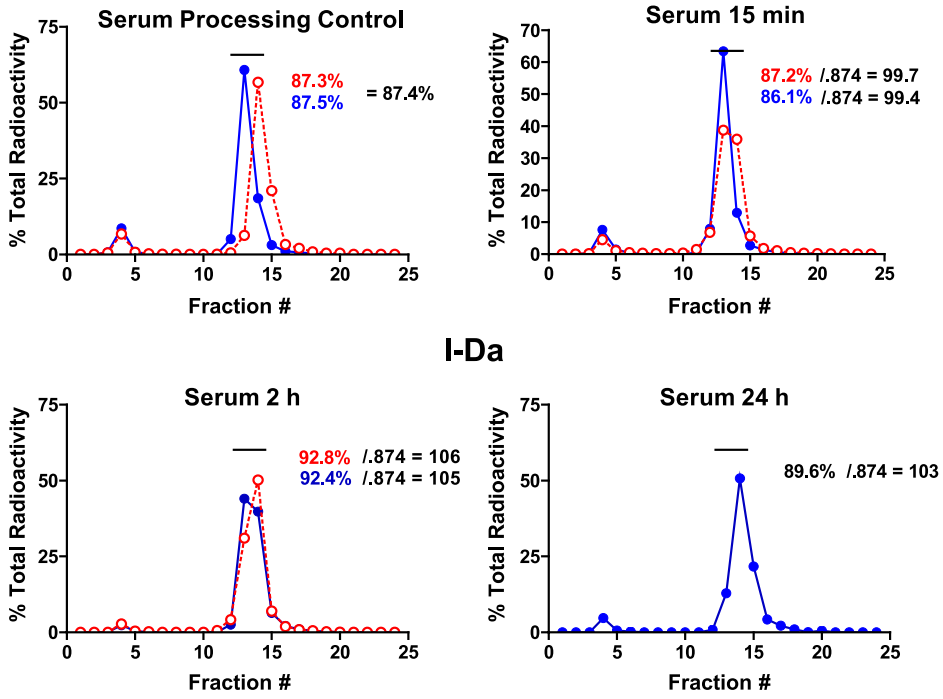

Fig. 2. HPLC results for I-darbepoetin alfa (I-Da): This compound in the blood showed the least rapid accumulation of degradation products. Radioactivity recovered from blood 15 min, 2 h, and 24 h after i.v. injection of I-darbepoetin alfa. The numbers to the right of the peaks shows the percent of material eluting in the region of I-darbepoetin alfa with the upper numbers correlating with the open symbol/broken line chromatograph and the lower numbers correlating to the closed symbol/solid line chromatograph.

blood-to-brain uptake could be measured. A comparison of the K_i's for I-murine erythropoietin and for I-Albumin showed they were different: $F(1,22)=10.2$, $P<0.005$.

Capillary depletion was performed with washout of the vascular space (Table 3, n=4–5/group). About 80% of the I-murine erythropoietin taken up by brain was recovered from the parenchymal space.

To determine whether I-murine erythropoietin was being transported across the blood–brain barrier by a saturable system, a short time curve (to 15 min) was performed by co-injection of I-murine erythropoietin with or without 10 μg/mouse of unlabeled I-murine erythropoietin. There was no statistically significant difference between the group which received no unlabeled murine erythropoietin (10.4±0.4, n=9) and the group that did (10.6±0.3, n=8).

Fig. 4 shows the results for %Inj/g for I-murine erythropoietin (n averaged 3 mice/time point). The half-time clearance of radioactivity from serum was 7.3 h. The mean value for %Inj/g across time was 0.0510±0.0102 for I-murine erythropoietin. The area under the curve was 31.7 (%Inj/g) min.

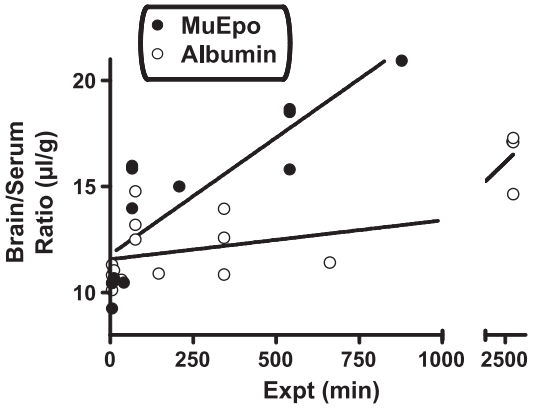

Fig. 3. Blood-to-brain passage of I-murine erythropoietin (MuEpo). The slope of the line measures the unidirectional rate of influx from blood to brain (K_i). The K_i for I-murine erythropoietin was 11.1±2.04 nl/g min when analyzed for the first 8 h after injection. The K_i for I-Albumin was 1.83±0.31 nl/g min when analyzed for the first 24 h after injection. See Results for values of n and r.

3.3. Human erythropoietin

I-Human erythropoietin was stable in brain and serum samples to 24 h (Table 1). The K_i for I-human erythropoietin

Table 2
Comparison of K_i (nl/g min) for I-erythropoietic agents and simultaneously injected I-Albumin calculated on data up to 8 or 24 h

	8 h	24 h
I-Murine erythropoietin/ I-Albumin	11.1±2.04/ NS	ND/ 1.83±0.31
I-Human erythropoietin/ I-Albumin	4.68±1.04/ 5.86±1.50	1.63±0.35/ 1.81±0.28
I-Darbepoetin alfa/ I-Albumin	NS/ 7.75±2.07	1.68±0.32/ 2.68±0.49

NS=not significant; ND=not done because of degradation of compound. See Results for n used to calculate each K_i.

Table 3
Capillary depletion results

	10 min Cap	10 min Paren	24 h Cap	24 h Paren
I-muEpo Erythropoietin	0.112±0.260	0.462±0.137	0.483±0.202	2.52±0.301
I-huEpo Erythropoietin	0.052±0.032	0.242±0.044	0.163±0.022	0.636±0.062
I-huEpo-repeat	0.030±0.026	0.152±0.041	0.152±0.106	1.83±0.22
I-Darbepoetin	0.064±0.010	0.158±0.035	0.618±0.102	1.73±0.206
I-Alb	0.210±0.192	0.485±0.062	0.992±0.146	1.75±0.183
I-Alb-repeat	0.304±0.027	0.616±0.185	1.93±0.875	2.09±0.293
I-Alb-repeat	0.262±0.013	0.472±0.053	0.728±0.103	1.22±0.103

Units are in microliters per gram. muEpo=murine erythropoietin; huEpo=human erythropoietin; Alb=albumin. Representative results for albumin are given. Values are means±S.E. (n=4–5 per cell).

was 1.63±0.35 nl/g min (n=17, r=0.763, P<0.001) in comparison to a K_i of 1.81±0.28 nl/g min (n=17, r=0.857, P<0.001) for co-injected I-Albumin (Fig. 5). A repeat of this experiment gave a K_i of 1.02±0.30 nl/g min (n=34, r=0.515, P<0.005) for I-human erythropoietin. No statistically significant correlation occurred between the brain/serum ratios and Expt for I-Albumin in this repeat experiment, indicating no measurable influx into brain. In neither case did the K_i for I-human erythropoietin and I-Albumin differ when the lines were compared statistically.

Capillary depletion was performed on two separate occasions at 10 min and 24 h after co-injection of I-human erythropoietin and I-Albumin. Vascular washout was performed for all data reported. The results of Table 3 show that some I-human erythropoietin was present in the brain parenchymal space 10 min and 24 h after i.v. injection.

Fig. 4 shows the % Inj/g for I-human erythropoietin (n averaged 4 mice/time point). The half-time clearance of radioactivity from serum was 7.3 h. The mean value for %Inj/g across time was 0.0227±0.00083. The area under the curve in units of (%Inj/g) min was 18.5 for I-human erythropoietin.

3.4. Darbepoetin alfa

Curves for thymidine incorporation as a measure of biological activity were fitted to a four-parameter model. The r^2 for all lines exceeded 0.993. The IC_{50} for all lines were in the range of 1.61 and 3.44 ng/ml with no difference between I-darbepoetin alfa and darbepoetin alfa.

I-Darbepoetin alfa was stable to 24 h in brain and blood (Table 1; Fig. 2). The K_i for I-darbepoetin alfa was 1.68±0.32 nl/g min (n=15, r=0.823, P<0.001) and the I-Albumin K_i was 2.68±0.49 (n=15, r=0.833, P<0.001); see Fig. 6. A statistical significance did not exist between these two K_i's. In a second study, I-darbepoetin alfa only was injected (no I-Albumin) and studied to 24 h; there was no significant correlation between exposure time and brain/serum ratios, indicating no accumulation by brain. Capillary depletion was performed once with washout (Table 3) and showed parenchymal uptake at 10 min and 24 h after injection.

A short time curve (to 15 min) was performed by injection of I-darbepoetin alfa with or without 10 μg/mouse of unlabeled darbepoetin alfa. Results were combined across time and the means compared by t-test. The mean for the group not receiving unlabeled darbepoetin alfa was lower (10.4±0.4, n=9) than the group receiving the unlabeled darbepoetin alfa (11.6±0.4, n=9). This difference was statistically significant: t=2.15, df=16, P<0.05.

The %Inj/g for I-darbepoetin alfa for all values across time was 0.0279±0.0161 (Fig. 4, n averaged 3 mice/time

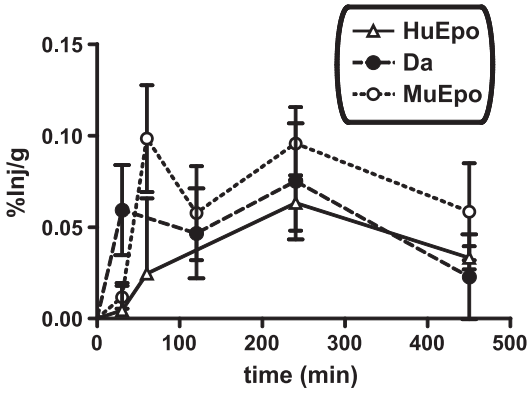

Fig. 4. Percent of the intravenously injected dose of the I-erythropoietic agents taken up per gram of brain (%Inj/g). Peak values were between 0.05 and 0.1 %Inj/g for each I-erythropoietic agent. HuEpo=human erythropoietin; MuEpo=murine erythropoietin. Da=darbepoetin.

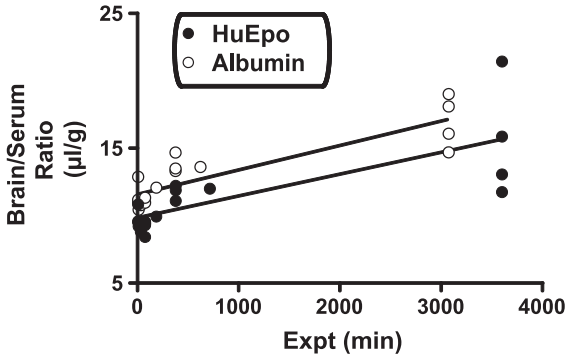

Fig. 5. Blood-to-brain passage of I-human erythropoietin (HuEpo). The slope of the line measures the unidirectional rate of influx from blood to brain (K_i). The K_i for I-human erythropoietin was 1.63±0.035 nl/g min. For the co-injected I-Albumin, the K_i was 1.81±0.28 nl/g min.

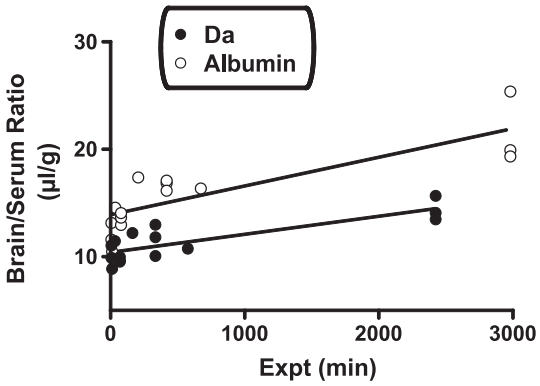

Fig. 6. Blood-to-brain passage of I-darbepoetin alfa (Da). The slope of the line measures the unidirectional rate of influx from blood to brain (K_i). The K_i for I-darbepoetin alfa was 1.68±0.32 nl/g min and the K_i for the co-injected I-Albumin was 2.68±0.49 nl/g min.

point). The half-time clearance of radioactivity from serum was 11.9 h. The area under the curve was 22.3 (%Inj/g) min.

4. Discussion

We found that the three erythropoietic agents studied here crossed the blood–brain barrier in measurable amounts. The erythropoietic agents were all extremely stable in blood and brain, slowly crossed the blood–brain barrier, and accumulated in the brain in amounts at which other cytokines are known to affect the CNS.

We used multiple-time regression analysis, a method specifically designed for the study of proteins which slowly penetrate the blood–brain barrier (Blasberg et al., 1983; Patlak et al., 1983), to determine the rate of passage of the radioactively labeled erythropoietic agents. We first assessed the stability of the radioactive compounds in brain and blood. With time, an increasing percentage of radioactivity in brain and blood will represent degradation products. Therefore, we first determined the stability of the I-erythropoietic agents in blood and brain up to 24 h after i.v. injection. This stability can be a separate issue from those of the clearance and the degradation of unlabeled proteins, especially if the protein–iodine bond is an early site of cleavage. The most stable compound was I-darbepoetin alfa in blood; in fact, no radioactive degradation products were seen during the 24 h period. The least stable was I-murine erythropoietin in brain. We limited our studies to those times when the radioactivity in both brain and blood was more than 50% intact I-erythropoietic agent. By so doing, we limited the potential bias in estimation of K_i to either a twofold increase (degradation in blood only) or twofold decrease (degradation in brain only). With these parameters, we were able to study I-human erythropoietin and I-darbepoetin alfa up to 24 h and I-murine erythropoietin for 8 h.

Having defined the time periods during which the radioactive compounds were stable, we then assessed them by multiple-time regression. This method plots brain/serum ratios against exposure time. The slope of the linear portion of the line measures the unidirectional influx rate from blood-to-brain (K_i); that is, the rate at which the compound is entering the brain. Exposure time rather than actual time is used as it is corrected for clearance of the compound from the blood. Not correcting for clearance would lead to an overestimation of influx. The three erythropoietic agents showed a number of similarities to each other. All crossed the blood–brain barrier, all crossed at a very slow rate and without the aid of a saturable component to blood-to-brain influx, all accumulated in the brain to about 0.05–0.1% of the injected dose, all had a peak in brain levels about 3 h after i.v. injection, and capillary depletion showed all crossed the full width of the capillary wall comprising the blood–brain barrier to enter brain tissue. Especially for I-human erythropoietin and I-darbepoetin alfa, the K_i was similar to that found for albumin. Because of the great sensitivity of multiple-time regression analysis, albumin can be used not as a vascular marker, but to measure its own influx by way of the extracellular pathways (Broadwell and Sofroniew, 1993; Balin et al., 1986). The similar rate of influx for the I-erythropoietic agents and I-Albumin suggests that the dominant mode of passage across the blood–brain barrier for the erythropoietic agents was also the extracellular pathways. This route is available to any substance circulating in blood, but penetration is so slow that it is usually deemed to be insignificant for the delivery of a therapeutic. Almost all currently used therapeutics are small molecules with large volumes of distribution and short half-lives; these pharmacokinetic characteristics are unfavorable for passage by way of the extracellular pathways. The ideal pharmacokinetic properties for a therapeutic to be delivered by way of the extracellular pathways would be a small volume of distribution within the body, a very long half-life in blood, and a slow rate of degradation. These were the characteristics of the erythropoietic agents studied here. The low blood-to-brain influx rate (K_i) of the erythropoietic agents, which was about 1/100th of that measured for those cytokines which cross the blood–brain barrier by a saturable transport system (Banks et al., 1995), was offset by their favorable pharmacokinetics. As a result, the percent of the injected dose taken up per gram of brain was about 0.05–0.1%. This is a value similar to that found for human interleukin-1 (Banks et al., 1991), a cytokine whose cognitive effects depend at least partially on its ability to cross the blood–brain barrier (Banks et al., 2001) and for pituitary adenylate cyclase activating polypeptide (Banks et al., 1993), a peptide shown to also be neuroprotective against stroke (Uchida et al., 1996). Therefore, despite the slow rate of influx, the percent of the administered dose accumulated by the brain of these compounds is in the range seen for agents which are centrally active. The possibility also exists that these agents release neuroprotective agents from within the CNS or even from the brain vasculature.

The major difference among the erythropoietic agents was likely caused by a species specificity. I-Murine

erythropoietin crossed the mouse blood–brain barrier faster than either I-human erythropoietin or I-darbepoetin alfa. This increased rate could have been caused by determining K_i over a substantially shorter time interval than for the other two erythropoietic agents. A requirement of the method used is that K_i be measured only during that interval when the relation between brain/serum ratios and Expt is linear. As the brain and blood compartments begin to approach equilibrium or, to state it in other terms, when brain-to-blood efflux starts to affect the accumulation which results from blood-to-brain influx, the relation between brain/serum and Expt begins to depart from linearity. The desire to avoid including data which is not part of the linearity must be balanced against the statistical advantages of calculating a linearity over as long a time period as possible. For substances which cross very slowly, it can be difficult to judge when linearity ends. The finding that in some cases the K_i for I-Albumin and for I-human erythropoietin was higher when data was assessed only up to 8 h rather than 24 h is likely caused by a subtle departure from linearity. Because of these considerations, the most informative way to evaluate these results is to compare compounds over the same time interval and to the simultaneously injected albumin. When compared based on 8 h data, I-murine erythropoietin entered the brain faster than the other I-erythropoietic agents or I-Albumin. Only the K_i for I-murine erythropoietin differed from that of its co-injected I-Albumin regardless of whether the lines for I-human erythropoietin, I-darbepoetin alfa, and their co-injected I-Albumin were analyzed for 8 or 24 h.

The increased penetration of I-murine erythropoietin in comparison to I-human erythropoietin and I-darbepoetin alfa was not based on a saturable component for influx. Previous work has suggested that human erythropoietin might cross the blood–brain barrier by way of a saturable transporter (Brines et al., 2000). This suggestion was reinforced by the clear findings that human erythropoietin was crossing the blood–brain barrier by some mechanism and that the brain capillaries possessed receptors for erythropoietin which might function as a transporter. Unfortunately, the saturation experiments used biotin to label human erythropoietin. Biotin has a major disadvantage for blood–brain barrier experiments in that it is rapidly transported across the blood–brain barrier (Spector and Mock, 1987; Shi et al., 1993). Therefore, even a small amount of degradation in blood can free enough biotin to cause a false signal.

In comparison to the lack of a saturable blood-to-brain component, the preliminary evidence suggests that a brain-to-blood efflux system may exist. The evidence for an efflux system was strongest for I-darbepoetin alfa, but was also suggestive for I-human erythropoietin. Such an efflux system would not only explain why the brain/serum ratio for I-darbepoetin alfa increased when unlabeled darbepoetin alfa was included in the i.v. injection, but also why its brain/serum ratios were the lowest in comparison to I-Albumin among the three erythropoietic agents. If this efflux were to be inhibited, which could occur with therapeutic amounts of erythropoietic agent, the uptake of I-human erythropoietin and of I-darbepoetin alfa should approach that of I-murine erythropoietin.

It is probable that the permeability of the blood–brain barrier to the erythropoietic agents will change after stroke. Several saturable transport systems are altered with various types of insults to the CNS (Pan et al., 1996; Pan et al., 1997; Banks et al., 1998; Pan et al., 2003) and stroke also induces functional changes at the blood–brain barrier (Brown and Davis, 2002; Rapoport, 1976). However, human erythropoietin most likely needs to be given within 6 h of stroke to be effective (Ehrenreich et al., 2002). The disruption of the blood–brain barrier following many types of CNS injury, even traumatic injury, may occur much later (Baldwin et al., 1996; Pan et al., 1997). In the case of stroke, evidence suggests that a significant disruption is demonstrable at 1–6 h after injury (Chi et al., 2003; Abraham et al., 2003) and that this disruption can be used to deliver therapeutics (Han et al., 2002; Vachon et al., 2002). As a result, uptake of erythropoietic agents by the injured brain region may be higher than those calculated here for the brains of normal animals.

In conclusion, erythropoietic agents were found to cross the blood–brain barrier very slowly by way of the extracellular pathways. The presence of an efflux system may play a secondary role in influencing the rate of influx, but extreme stability in the brain and blood were the main factors in determining the amount of i.v. injected material accumulated by brain. The amounts of each of the three erythropoietic agents taken up by brain are similar to the amounts taken up for other cytokines known to exert effects on the brain by crossing the blood–brain barrier. We conclude that erythropoietic agents cross the blood–brain barrier by way of the extracellular pathways in amounts that could account for their neuroprotective effects.

Acknowledgments

Supported by VA Merit Review, Amgen, R01 NS41863, and R01 AA12743. We thank Hiko Kohno, Xin Wei, and Sheila Scully for technical assistance.

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